A microtiter plate-based quantitative method to monitor the growth rate of dermatophytes and test antifungal activity

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Abstract

Dermatophytosis is one of the most common superficial fungal infections, which is mainly caused by filamentous fungi such as *Trichophyton* species. A challenging aspect in dermatophyte research is the lack of a straightforward method to measure the rate of growth, in particular when growing dermatophytes in small volumes such as in microtitre plates. However, one characteristic of dermatophytes is their ability to produce compounds such as ammonia that make the growth medium more alkaline. The objective of this study was to test whether the change in pH in a liquid medium, colourimetrically established using the indicator phenol red, was linearly and directly proportional to the growth rate for *Trichophyton rubrum* and *Trichophyton interdigitale*. The changes in the colour determined by the phenol-red based assay showed a good correlation with the amount of fungal biomass over an incubation period of 24-120 hours. The functionality of the phenol red assay was also validated in experiments on the growth of *T. rubrum* in the presence of antifungals. The changes in colour showed a clear dose-response relationship compounds and enabled determination of the minimum inhibitory concentration. The phenol red assay is thus a simple and straightforward assay to monitor the rate of growth of *Trichophyton* spp. and test antifungal activity.

1. Introduction

Dermatophytosis is one of the most common fungal infections of keratinised tissues such as nails, hair and skin, with about 20 to 25% of the world population being infected (Havlickova et al., 2008). They are most commonly caused by dermatophytes, which are fungi that can degrade keratin. *Trichophyton rubrum* and *Trichophyton interdigitale* are the two most common causative agents, causing over 90% of dermatophyte infections in 2005 in the UK (Borman et al., 2007).

With bacteria, growth in liquid cultures can be monitored easily using, for instance, the optical density (OD) of cultures. This technique does not work well for moulds due to their filamentous and heterogeneous morphology in liquid cultures. For such fungi, one could measure radial growth on agar plates, and for liquid medium, a traditional method would be determining the mycelium dry weight. However, the latter method is time-consuming due to the relatively slow growth rate of most filamentous fungi. Furthermore, it is also challenging to implement in the case of multi-replicate assays and when growing fungi in small volumes such as in microtitre plates (Arima and Uozumi, 1967; Granade et al., 1985; Matcham et al., 1984; Taniwaki et al., 2006). An alternative to dry weight measurements is by staining the fungal biomass with a dye such as crystal violet, and then measure the absorbance of the dye, but this is still fairly laborious and involves several steps (Costa-Orlandi et al., 2014).

An alternative approach to measuring fungal biomass is to use colourimetric assays that monitor metabolic activity, such as those based on resazurin or the tetrazolium salts 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and these have been applied to, for instance, antifungal susceptibility testing with *Aspergillus* species (Jahn et al., 1996; Meletiadis et al., 2002; Monteiro et al., 2012). However, dermatophytes prefer a mildly acidic environment for the initial stages of infection, as a number of important virulence factors such

as proteases have optimal activity at the pH that matches that of the skin (pH 5.5) and nail (pH 5.2) (Martinez-Rossi et al., 2012; Matousek and Campbell, 2002; Murdan et al., 2016). During growth of dermatophytes, degradation of keratin or other proteins leads to release of ammonia, resulting in a shift towards an alkaline pH (Mercer and Stewart, 2019). The initial low pH reduces the adequacy of the MTT and XTT cell viability assays that require a neutral pH (pH 7.4). Indeed, it was shown that the acidic environment shifts the absorbance and reduces the activity of formazan-producing enzymes (Grela et al., 2018; Johno et al., 2010; Plumb et al., 1989). Moreover, the resazurin assay monitors not only cell viability but also functions as a pH indicator (Lancaster and Fields, 1996), and false negative results may therefore result from the shift towards alkaline pH. Therefore, better and easier assays to monitor growth and viability of dermatophytes are desirable and would be useful to study e.g. antifungal susceptibility.

The ability of dermatophytes to producing ammonia has been exploited to develop a simple and rapid dermatophyte test medium (DTM) to diagnose the presence of dermatophytosis by incorporating the pH indicator phenol red, which changes colour from yellow to red under alkaline conditions (Taplin et al., 1969). This colour change is characteristic for dermatophytes such as *T. rubrum* and *T. interdigitale* and aids in their identification It should be noted, however, that some non-dermatophytes may also cause a colour change in DTM, leading to the introduction of an improved Dermatophyte Identification Medium (Salkin et al., 1997; Mesquita et al., 2016; Gromadzki et al., 2003). Here we developed a simple method that uses phenol red to measure the rate of fungal growth in liquid culture media, and also demonstrate its usefulness to study antifungal susceptibility of dermatophytes. The phenol red assay was validated by comparison with quantification of the biomass, as determined using crystal violet (Costa-Orlandi et al., 2014). Compared with other fungal growth and viability assays, this assay is inexpensive and straightforward, with minimal post-processing that avoids artefacts.

2. Materials and methods

2.1 Strains and culture conditions

Trichophyton species used in this study were *T. interdigitale* (ATCC 9533) and *T. rubrum* (ATCC 28188), obtained from Fisher Scientific (Loughborough, UK). To isolate microconidia, strains were cultured on potato dextrose agar (Sigma-Aldrich, St. Louis, MO, USA) for 15 days at 30°C to induce full sporulation. Conidia were harvested with sterile 1% Tween-20 (Fisher Scientific), filtered through a sterile cell strainer (40 µm) and resuspended in sterile Milli-Q water. The conidia were aliquoted and stored at -20°C and aliquots were used within two weeks, during which no significant reduction in viability was observed.

2.2 Fungal growth assay

The fungal growth assay was performed by dispensing 200 µL of Sabouraud dextrose broth (SDB) with phenol red (0.002%) in 96-well flat-bottom microtitration plates (Costar, Corning, N.Y., USA) and adding microconidia to each well with a final concentration of 1×10^6 CFU ml^{-1}. The plate was then sealed with parafilm and incubated for 24-120 hrs on a microplate shaker (150 rpm) at 30°C. Finally, the liquid from each well was transferred into a new microtitration plate and then read in UV/vis multiplate reader (CLARIOstar Plus, BMG Labtech, Aylesbury, UK) at a wavelength of 560 nm.

2.3 Quantification of biomass by crystal violet staining

Trichophyton spp. biomass quantification was done by crystal violet staining, as described by Costa-Orlandi et al. (2014), with some slight modifications. Briefly, after growth of the fungi in 96-well plates in SDB, the solution from each well was removed, the fungal biomass at the bottom of each well was washed twice with PBS, and then the plate was dried in an oven for 20 min at 60°C. The biomass in each well was then stained for 5 min with 150 µL of 0.1%

crystal violet solution. The solution was carefully removed, and wells were submerged three times in trays with cold tap water to remove excess stain. After that, 200 µL of 95% ethanol was added to each well to dissolve the crystal violet. After mixing thoroughly, the solution was transferred to a new microtitration plate and read in a UV/Vis multiplate reader at a wavelength of 595 nm.

2.4 Antifungal susceptibility test

The potential of an antifungal susceptibility test based on the phenol red assay was tested with nystatin and EDTA. 200 µL of SDB with phenol red (0.002%) and different concentrations of EDTA or nystatin (0.1-256 µg/mL) was dispensed in 96-well plates, followed by adding *T. rubrum* conidia to each well at a final concentration of 1×10^6 CFU ml^{-1}. The plate was incubated for 72 hours on a microplate shaker (150 rpm) at 30°C, after which the solutions from each well were transferred to a new plate and the absorbance at a wavelength of 560 nm was determined using a multiplate UV/vis spectrometer.

To apply the phenol red assay by addition after growth, cells were grown as above in SDB (without phenol red) in the presence of antifungal compounds (nystatin, cycloheximide, terbinafine hydrochloride or clotrimazole). After 72 hours the supernatant was transferred to a new plate containing 2 µl 0.2% phenol red (in DMSO) in each well.

2.5 Calculation and statistical analysis

The results were presented as the mean ± standard deviation. Since the response of absorbance is different in each assay, the results from the phenol red and crystal violet biomass assays were expressed using the following equation (Eq.1) and plotted against incubation times.

$$\%\ \text{percentage change} = \frac{(\text{Sample at 120 hrs}_{\text{Abs}} - \text{Blank}_{\text{Abs}})}{(\text{Sample}_{\text{Abs}} - \text{Blank}_{\text{Abs}})} \times 100\% \qquad (Eq.1)$$

The slope and intercept of these linear regressions were compared by analysis of covariance (ANCOVA), performed by GraphPad Prism software. Statistical significance was considered for *p*-values less than 0.05.

The growth inhibition rate of antifungal susceptibility test was calculated by the equation below (Eq.2), and the data were plotted against concentrations of the antifungal agents. The minimum inhibitory concentration (MIC) is defined as the lowest concentration that gives at least 90% growth inhibition as measured using the phenol red assay.

$$\%\ \text{growth inhibition} = \left[1 - \frac{(\text{Sample}_{\text{A560}} - \text{Blank}_{\text{A560}})}{\text{Control}_{\text{A560}}}\right] \times 100\% \quad (Eq.2)$$

3. **Results**

3.1 Correlation between the phenol red growth assay and biomass quantification

The pH of *Trichophyton* cultures grown in Sabouraud broth shifted from 5.5 to 8.0 throughout 120 hrs (data not shown). The indicator phenol red has an absorption peak at 430 nm at low pH, whereas the absorption maximum shifts to 560 nm between pH 6.8-8.2, which results in the change from yellow to red in the culture medium. We examined whether this colour change could be used to measure the growth of *T. rubrum* and *T. interdigitale* by following the absorbance of the culture supernatant containing phenol red at 560 nm in the period 24-120 hours after inoculation. Incubation times shorter than 24 hours showed no change in the colour of phenol red, while with longer periods of time there was so much biomass that it became difficult to extract the liquid from the wells. The results from the phenol red assay were compared with the data on biomass formed as determined using crystal violet staining. As shown in Fig 1A and 1B, the two assays show very similar results, with no significant difference found between the slopes or intercepts of the two linear regressions as determined by ANCOVA. Very similar results were also obtained when comparing the phenol red assay with release of protein and DNA after treatment in lysis buffer (data not shown). However, complete lysis was difficult to achieve; as it was unclear whether the level of lysis was similar in all stages of growth, these results were less conclusive.

3.2 Application of phenol red assay to antifungal susceptibility testing

To further test the usefulness of the phenol red assay, the growth of *T. rubrum* was determined in the presence of antifungal compounds. Two known growth inhibitors, the antifungal nystatin and the chelator EDTA, were used to evaluate the susceptibility test. As shown in Fig 2, the growth inhibition effect was reflected in the results of the assay, and a clear dose-response relationship between the growth and the concentration of inhibitors was observed. The curves

in Fig. 2 were used to determine the MIC for nystatin and EDTA, which were 3 µg/mL and 48 µg/mL, respectively.

We also tested whether the susceptibility testing can be done by adding a stock solution of phenol red after fungal growth. As shown in Figure 3, we could visualise inhibition of *T. rubrum* growth in the presence of terbinafine (inhibition at <0.01 µg/mL), clotrimazole (<0.1 µg/mL and nystatin (<10 µg/mL), whereas no inhibition was observed with cycloheximide. The latter antifungal is used in DTM as it inhibits saprophytic fungi whereas dermatophytes are resistant to cycloheximide.

4. Discussion

Given their relevance to human health, improved techniques to study the antifungal drug therapies are required. Due to the heterologous morphology of filamentous fungi, the typical measurements of microbial growth and viability such as optical density and colony counting cannot be used. Instead, mycelium dry weight is the most common method to measure the filamentous fungal growth. However, this is time-consuming, and it is difficult to process multi-replicate assays, in particular when working with small volumes (Arima and Uozumi, 1967; Granade et al., 1985; Matcham et al., 1984; Taniwaki et al., 2006).

Phenol red is a commonly used pH indicator to monitor the pH of the cell culture media, based on a gradual transition in colour following pH shifts from acidic to alkaline, and *vice versa*. Based on this, DTM (containing phenol red) was developed to differentiate dermatophytes from other fungi as the only the former cause an alkaline shift on the pH of the medium to 8-9, by deaminating amino acids to form ammonia as a by-product (Kunert, 2000; Monod, 2008; Taplin et al., 1969). In this study, we expand the usage of phenol red so it can be used as an assay to measure fungal growth and to determine antifungal activity in liquid cultures. The phenol red growth assay was validated by measuring the fungal growth of *T. rubrum* and *T. interdigitale* in SDB based on the biomass between 24-120 hours of growth. Thus, alkalinisation of the medium as a result of utilising nitrogen sources is directly correlated to fungal growth, demonstrating that the phenol red assay is a reliable and easy method to measure growth of *Trichophyton* spp.. It should be noted that some non-dermatophytic fungi such as *Hisptoplasma capsulatum* and *Blastomyces dermatitidis* also cause a similar change of colour in media containing phenol red (Salkin, 1973). It is thus conceivable that the method developed here may be employed for a number of these fungi, but we have not tested this.

The phenol red growth assay can overcome the disadvantages of various methods used to measure fungal growth. Apart from traditional methods such as dry weight measurements,

crystal violet staining has been used to study biofilm formation of *T. rubrum* (Costa-Orlandi et al., 2014) and the MIC of various antifungal compounds on filamentous biomass (Kvasničková et al., 2016; Mowat et al., 2007). However, the crystal violet staining requires fungi to adhere to the surface of the culture plates, and the biomass of some filamentous fungi may be removed easily during the washing steps. The phenol red growth assay involves a simple procedure and does not require any washing steps. We also showed that a phenol red stock solution can be added after growth and can be used to visualise inhibition of several antifungal agents. The values obtained corroborated earlier studies that determined the MIC for these antifungal agents (Gupta and Kohli, 2003, Shadomy, 1971, Adimi et al., 2013, Agbulu et al 2015).

The antifungal susceptibility using phenol red was demonstrated by studying the inhibitory effect of nystatin, a polyene antifungal agent, and EDTA, a chelating agent and metalloprotease inhibitor to *T. rubrum* (Sen, 1964). Notably, the MIC for nystatin was the same as found in a study that determined this using SDB slant cultures (Agbulu et al., 2015). The sigmoidal dose-response curves were established after 72 hours incubation, a time point is commonly used in studying the fungal growth of dermatophytes, and provides an optimum response of the susceptibility (Costa-Orlandi et al., 2014; Smijs et al., 2008). Furthermore, at 72 hours it was still easy to extract liquid from the wells, which became more difficult with longer incubation times. Thus, the phenol red assay provides an alternative method to determine MIC values. The method does not replace existing standardised methods to determine antifungal activity of filamentous fungi (Johnson, 2008), as the phenol red assay is limited to dermatophytes only, but it is nevertheless a useful addition because of its ease and simplicity. In conclusion, the phenol red growth assay provides a reproducible and cheap method that simplifies the study of dermatophytes and enables antifungal susceptibility testing.

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Fig 1. Measurement of fungal growth of *T. rubrum* **(A)** and *T. interdigitale* **(B)** in SDB between 24-120 hours *via* crystal violet biomass staining. Each assay was completed with the phenol red viability assay, and their rate of the percentage change versus the incubation times are did not show a significant difference (ANCOVA – A-B, *ns*, $n = 3$).

Fig 2. Dose-response curves illustrating the antifungal susceptibility activities of nystatin **(A)** and EDTA **(B)** against *T. rubrum* in SDB using the phenol red growth assay after 72 hours of incubation (A: $R^2 = 0.9955$; B: $R^2 = 0.9731$; $n = 3$).

Fig 3. Antifungal susceptibility testing by adding phenol red after growth. The antifungals used were terbinafine (Te), clotrimazole (Cl); cycloheximide (Cy), and nystatin (Ny), with the concentrations used indicated above the panels.

345 Figure 1

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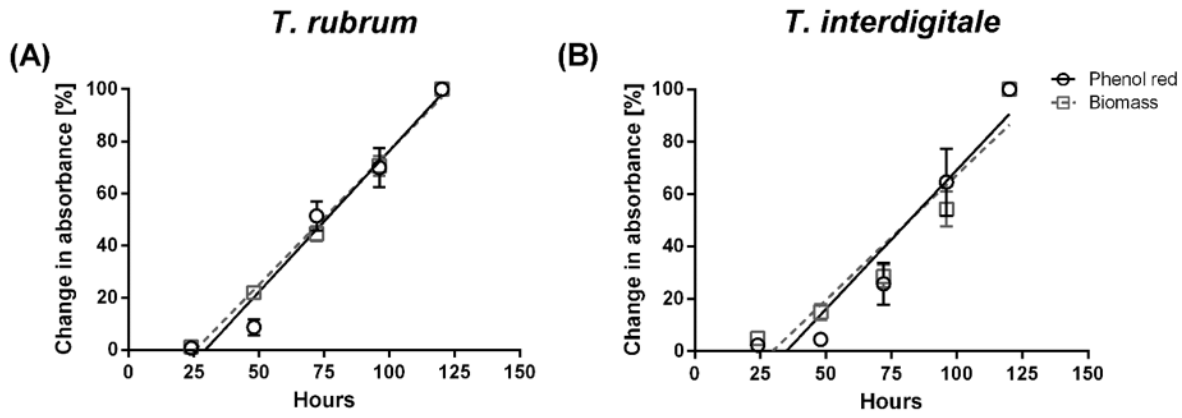

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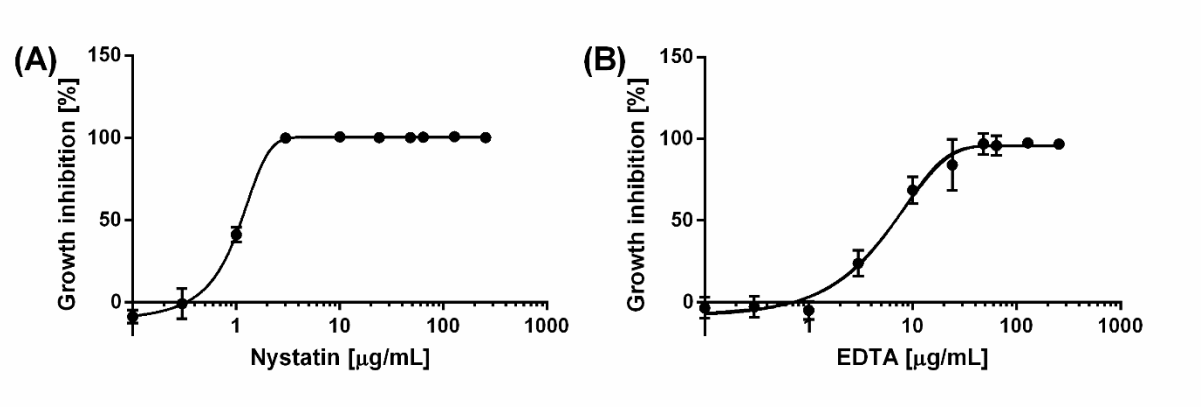
Figure 2


(A)
Growth inhibition [%]
150
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Nystatin [μg/mL]
(B)
Growth inhibition [%]
150
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1
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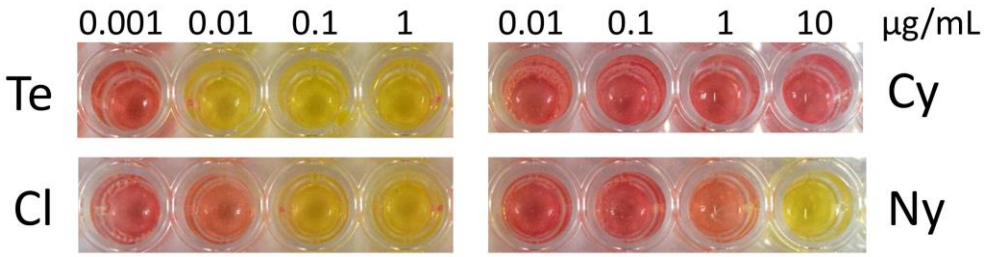
EDTA [μg/mL]

352 Figure 3



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